

Extraction and purification of violacein from *Yarrowia lipolytica* cells using aqueous solutions of surfactants

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Abstract

BACKGROUND: The demand for colorants from natural bio-based sources is increasing. Violacein is a natural purple-blue hydrophobic pigment with interesting bioactivity whose expression in genetically modified *Yarrowia lipolytica* production was successfully achieved.

RESULTS: In this work, several surfactants were tested in the extraction of violacein from *Y. lipolytica* cells, and the operational conditions were optimized to maximize the extraction yield. After the optimization, the purification of violacein using aqueous biphasic systems (ABS) composed of Tween 20 and cholinium-based ionic liquids (ILs) was pursued. The ABS were characterized and applied in the separation of violacein from contaminant proteins, reaching a maximum selectivity of 155 with violacein being fully concentrated in the Tween 20-rich phase, with 80% of the contaminant proteins present in the extract being removed.

CONCLUSION: This work led to a conceptual downstream process based on a first step of (solid–liquid) extraction and a second step addressing the separation of violacein from contaminant proteins using ABS.

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Supporting information may be found in the online version of this article.

Keywords: solid–liquid extraction; aqueous biphasic systems; violacein; surface-active compounds; ionic liquids; *Yarrowia lipolytica*

INTRODUCTION

Violacein is a natural purple-blue hydrophobic pigment¹ with interesting biological activities such as antitumoral, antiparasitic, antifungal, antiviral, antiprotozoal, antioxidant, antiulcerogenic and immunomodulatory.² In the past few years, violacein has also been reported to have analgesic and antipyretic properties with responses similar to those exerted by morphine and paracetamol respectively.² The biosynthesis of this indole derivative is associated with a secondary metabolic pathway that involves L-tryptophan and requires the joint action of five enzymes (via ABCDE operon) for its efficient biosynthesis.³

Yarrowia lipolytica is an aerobic dimorphic non-pathogenic yeast that has raised the interest of many researchers owing to its great biotechnological potential associated with many kinds of metabolites.⁴ Violacein is not produced by wild-type *Y. lipolytica*. To induce the production of this pigment, the yeast may be genetically modified. After production, the release of the pigment from the cells becomes of utmost importance, for example by the integration of a cell wall disruption step for the extraction. The available methods for intracellular compound release are normally divided into two main groups, mechanical and non-mechanical techniques.⁵ Mechanical techniques (bead mill, homogenization and ultrasonic treatment) are easy to scale up. However, they present a non-selective character and can negatively affect the biological activity of the target compounds and

the downstream process owing to the finer cell debris resulting from the high degree of disruption. The high energy consumption is another negative point that should be highlighted. On the other hand, non-mechanical techniques (electrical, physical, chemical and enzymatic) are more selective and gentler. Nonetheless, these methods are often limited to laboratory scale owing to the low efficiency of physical methods (osmotic shock and thermolysis) and economic constraints.⁶

Conventional solid–liquid extractions use volatile solvents such as acetone, methanol, ethanol or chloroform, to mention just a few.^{7–9} Their use can contribute to increase the environmental footprint of the processes (with the exception of ethanol that is non-toxic and biodegradable).^{10,11} Alternative solvents such as ionic liquids (ILs) and surfactants have emerged aiming to

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overcome the major drawbacks present in traditional processes. Surfactants are a wide group of chemicals with amphiphilic nature used for a diversity of applications, including to achieve the dispersion in water of poorly soluble compounds and for the extraction of biocompounds from biomass.^{12,13} Aqueous solutions of surfactants have some advantages over other solvents since they require using lower concentrations, leading to cheaper and more sustainable processes.¹⁴

Various studies^{15,16} have shown that surfactants can disrupt the cell membrane when strong hydrophobic interactions are present. The spontaneous insertion of the surfactant alkyl chain in the lipid bilayer causes swelling of the cell membrane, leading to lipid bilayer disintegration. Hydrophobic anions can further intrude into the membrane along with cations with big alkyl side chains, leading to easier cell membrane breakdown.¹⁷

Considering the low selectivity obtained during (solid–liquid) extraction, different strategies are being explored to meet the needs for further purification.

Aqueous biphasic systems (ABS) are purification technologies that are cost-effective, simple, versatile, easy to scale-up and to recycle, biocompatible and tunable.^{18,19} They have two aqueous phases, which are formed by mixing two incompatible water-soluble solutes in water above certain concentrations.²⁰ Although polymer pairs or a polymer and a salt are the most used,²¹ multiple combinations have been proposed in recent decades. Examples include the use of organic solvents,²² surfactants,^{16,23} carbohydrates,²⁴ amino acids²⁵ and ILs.²⁶

Over the last few decades, ILs have grown to be a subject of widespread research as promising alternatives to common organic solvents. ILs fit a class of salts with low symmetry of the composing ions and low charge density that precludes their organization into a crystal lattice, which generally translates into low melting temperatures, usually below 100 °C.²⁷ ILs possess some unique properties (negligible vapor pressure at atmospheric conditions, strong solvation ability for a wide variety of compounds, adequate thermal and chemical stability) and thus have interesting uses as solvents in a wide variety of applications. Considering their beneficial general traits, cholinium-based ILs can be further highlighted owing to their great biodegradability and low toxicity.^{28,29} Moreover, this family of ILs is considered as more biocompatible, cheap and has been reported as an essential family to develop more sustainable downstream processes.³⁰

ABS were shown to be highly performant and selective if properly chosen.^{21,31} Owing to their aqueous environment, they are recognized as an appropriate technology for the recovery and purification of biomolecules and other biologically active substances.³² Cholinium-based IL features combined with the potential benefits of ABS lead to high-performance separations besides other advantages such as quick phase separation and low viscosity favoring mass transfer.^{33,34} As mentioned, ABS composition is markedly versatile. This technology will allow the design of an integrated process, bridging the solvents used in the extraction and purification steps of an industrially favorable process.

The focus of this work was the optimization of the extraction of violacein produced by genetically modified *Y. lipolytica* cells and its further purification. In this sense, various solvents (including ILs, common surfactants and conventional solvents) were tested and several conditions were studied, namely the number of extractions, the solid–liquid ratio and the time of extraction. After the most promising system was unveiled, the purification of the pigment was investigated through the application of ABS using cholinium-based ILs. Lastly, violacein was retrieved from the

phase-former using a back-extraction step. Ultimately, a complete process for the extraction and purification of violacein is proposed.

MATERIALS AND METHODS

Materials

Strain construction and biomass production

The violacein pathway consists of five genes, VioA, VioB, VioC, VioD and VioE. Genes were amplified from an *Escherichia coli* operon kindly provided by Cyrille Pauthenier.³⁵ Constructions of the violacein VioABE and VioCD cassettes were done following the Golden Gate Assembly (GGA) strategy dedicated for *Y. lipolytica* according to Celińska *et al.*³⁶ and Larroude *et al.*³⁷ The upper part of the pathway consisting of gene VioA–VioB–VioE was assembled by GGA giving rise to the VioABE cassette with the *URA3ex* marker (ABE-12), and the lower part was assembled by GGA giving rise to the VioCD cassette with the *LEU2ex* selection marker (CD-9). Both plasmids had ampicillin resistance, as presented in supporting information Fig. S1.

Po1d strain (*MATa*, *ura3-302*, *leu2-270*, *xpr3-322* + *pXPR2-SUC2*) was co-transformed with the two expression cassettes, obtained after *NotI* digestion of plasmids ABE-12 and CD-9 (*URA3ex*–VioABE and *LEU2ex*–VioCD respectively), using the lithium acetate method.³⁸ Transformants were selected on minimal medium YNB. The resulting strain was named JMY7019 (Po1d, *URA3ex*–VioABE, *LEU2ex*–VioCD).

For biomass production, JMY7019 cells were pre-grown in minimal medium YNB supplemented with tryptophan (25 mg L^{−1}) for 2 days at 28 °C with constant shaking. YNB was composed of 1.7 g L^{−1} yeast nitrogen base (without amino acids and ammonium sulfate, YNBww, Difco), 5 g L^{−1} NH₄Cl and 50 mmol L^{−1} KH₂PO₄–Na₂HPO₄ buffer (pH 6.8). The pre-culture was used to inoculate minimal enriched medium YNB_D₃YP consisting of YNB with glucose (30 g L^{−1}), yeast extract (0.5 g L^{−1}) and peptone (0.5 g L^{−1}) supplemented with tryptophan (25 mg L^{−1}) for 5 days at 28 °C with constant shaking.

All restriction enzymes used in this study were purchased from New England Biolabs (NEB), while Q5 high-fidelity DNA polymerase (NEB) or GoTaq DNA polymerase (Promega, Charbonnières-les-Bains, France) was used for polymerase chain reaction (PCR) amplification. QIAquick Gel Extraction Kit (Qiagen, Courtaboeuf, France) and QIAprep Spin Miniprep Kit (Qiagen) were used to purify PCR fragments and to extract plasmids from *E. coli* respectively. All reactions were performed according to manufacturers' instructions.

Chemicals

The ethanol used was supplied by Fisher Scientific (Analytical Reagent Grade). The non-ionic surfactants used were Tween 20 (purity 99%) and Triton X-114 (purity 99%), while the anionic surfactant tested was sodium dodecyl sulfate, SDS (purity 99%), all of them provided by Acros Organics. The following ILs were used: 1-butyl-3-methylimidazolium chloride, [C₄mim]Cl (purity 99%, Iolitec); 1-hexyl-3-methylimidazolium chloride, [C₆mim]Cl (purity 98%, Iolitec); tetrabutylphosphonium chloride, [P_{4,4,4,4}]Cl (purity 95%, Iolitec); tetrabutylphosphonium bromide, [P_{4,4,4,4}]Br (purity 95%, Iolitec); tetrabutylammonium chloride, [N_{4,4,4,4}]Cl (purity 97%, Sigma-Aldrich); (1-hexyl)trimethylammonium bromide, [N_{1,1,1,6}]Br (purity 98%, Alfa Aesar); 1-methyl-1-propylpiperidinium chloride, [C₃mpip]Cl (purity 99%, Iolitec); 1-butylpyridinium chloride, [C₄py]Cl (purity 98%, Iolitec); 1-butyl-

1-methylpyrrolidinium chloride, [C₄m_{pyr}]Cl (purity 99%, Iolitec); 1-methyl-3-octylimidazolium chloride, [C₈mim]Cl (purity 99%, Iolitec); 1-decyl-3-methylimidazolium chloride, [C₁₀mim]Cl (purity 98%, Iolitec); 1-dodecyl-3-methylimidazolium chloride, [C₁₂mim]Cl (purity >98%, Iolitec); 1-methyl-3-tetradecylimidazolium chloride, [C₁₄mim]Cl (purity 98%, Iolitec); 1-hexadecyl-3-methylimidazolium chloride, [C₁₆mim]Cl (purity >98%, Iolitec); 1-dodecyltrimethylammonium bromide, [N_{1,1,1,12}]Br (purity 99%, Alfa Aesar); 1-tetradecyltrimethylammonium bromide [N_{1,1,1,14}]Br (purity 98%, Alfa Aesar); hexadecylpyridinium chloride monohydrate, [C₁₆py]Cl·H₂O (purity 99–102%, Sigma); tributyltetradecylphosphonium chloride, [P_{4,4,4,14}]Cl (purity 95%, Iolitec); cholinium acetate, [Ch][Ac] (purity >99%, Iolitec); cholinium dihydrogenphosphate, [Ch][DHP] (purity >98%, Iolitec); cholinium dihydrogencitrate, [Ch][DHC] (purity 99.0%, Sigma); cholinium chloride, [Ch]Cl (purity 98%, Acros Organics); and cholinium bicarbonate [Ch][Bic] (80% in water, Sigma). The chemical structure and abbreviation of each compound are depicted in supporting information Fig. S2. Toluene (purity 99.8%), acetone (purity 100%) and ethyl acetate (Analytical Reagent Grade) were provided by Fisher Scientific. 1,5-Pentanediol (purity 97%) and 2-methyl-tetrahydrofuran (2-methyl-THF) (purity 99%) were supplied by Alfa Aesar. *n*-Hexane (purity 99%) was acquired from Carlo Erba. L(–)-Menthol (99.5% purity) and thymol (99.0% purity) to prepare the mixture 1:1 (v/v) menthol/thymol were obtained from Acros Organics and their purity confirmed by ¹H and ¹³C nuclear magnetic resonance (NMR) analysis. 3-Pentoxypentan-1,2-diol [5.0.0], 3-hexyloxypentan-1,2-diol [6.0.0] and 1,3-dipropoxypropan-2-ol [3.0.3] were synthesized in-house following the protocol previously reported by Leal-Duaso *et al.*^{39,40} The precursors glycidol (96 wt% pure) and epichlorohydrin (>99.0 wt% pure) were purchased from Sigma-Aldrich. Sodium hydroxide (>90.0 wt% pure) was supplied by Scharlab. Propan-1-ol, pentanol and hexanol were purchased from Alfa Aesar. The alcohols were dried and distilled over calcium hydride (>93.0 wt% pure, Acros Organics) prior to use. The water used was doubly distilled, passed across a reverse osmosis system and further treated with a Milli-Q Plus 185 water purification apparatus.

Methods

Screening of surfactant and non-surfactant compounds

The chemical method for cell disruption was adapted from the literature.^{41,42} The cell suspension was homogenized with aqueous solutions of surfactants to test their ability to release violacein. A large array of 21 surfactant (cationic, anionic and non-ionic) and non-surfactant compounds were tested to assess their ability to permeate the cell membrane, releasing the violacein. Two control extractions using ethanol and water were also performed.

The screening was performed at a fixed surfactant concentration of 250 mmol L^{−1} and solid–liquid ratio (SLR) of 0.025 (mass of wet cells (g) per volume of solvent (mL)). Briefly, the biomass was placed in contact with aqueous solutions of surfactant and non-surfactant agents, and the samples were subjected to constant stirring (50 rpm) for 30 min in an orbital mixer at room temperature. The extractions performed in this work were carried out in the dark to better preserve violacein stability.⁴³ After the extraction, the samples were centrifuged (13400 g, 20 min) in a Micro Star 17 centrifuge (VWR) to efficiently separate the cell debris from the aqueous solutions rich in violacein. The resultant pellet was discarded while the violacein-rich aqueous supernatant was collected and its absorption spectrum determined between

200 and 700 nm in a UV–visible microplate reader (Synergy HT, BioTek). The violacein content was quantified at the violacein maximum peak of absorbance observed, 571 nm. All extractions were carried out in duplicate, the results being presented as the average of the two. The yield of extraction was calculated according to Eqn (1):

$$\text{Yield of Extraction (mg g}^{-1}\text{)} = ([\text{violacein}] \times \text{volume}) / \text{weight} \quad (1)$$

where '[violacein]' is the concentration of violacein in the medium (mg mL^{−1}), 'volume' is the volume of extract collected (mL) and 'weight' is the weight of wet cells tested (g).

Optimization of solid–liquid extraction

The solid–liquid extraction step was further optimized aiming to achieve the maximum extraction yield of violacein in just one step. For that purpose, the surfactant compounds with the best cell-disrupting performance were selected to evaluate the effects of the SLR (0.006–0.05), the extraction time (30–240 min) and the surfactant concentration (50–325 mmol L^{−1}). In addition, successive extractions using specific solvents selected during the experiments were also tested. These consecutive extractions were carried until no further peaks were detected at 571 nm.

Purification of violacein using ABS

Phase diagrams. The ternary phase diagrams were determined using the cloud point titration method at 298 K and atmospheric pressure. The experimental procedure adopted has been validated in previous reports.⁴⁴ Aqueous solutions of Tween 20 at ~90 wt% and aqueous solutions of the cholinium-based ILs with concentrations varying between 60 and 80 wt% were prepared gravimetrically (within ±10^{−4} g). The repetitive dropwise addition of the aqueous solution of Tween 20 + [Ch]X–water mixture was carried out until a cloudy biphasic mixture was discerned. Subsequently, distilled water was added dropwise until the mixture became translucent, reaching the monophasic region. This procedure was repeatedly performed under constant stirring until no more cloud points were observed. The composition of the systems after the addition of each component was determined by weight quantification (within ±10^{−4} g). The experimental binodal curves were fitted by the following equation (Eqn (2)) proposed by Merchuk *et al.*⁴⁵

$$[\text{Tween 20}] = A \exp[B([\text{Ch}]X)^{0.5} - C([\text{Ch}]X)^3] \quad (2)$$

where [Tween 20] and [Ch] are respectively the surfactant and IL weight fraction percentages for ABS composed of Tween 20 + [Ch]X + H₂O and *A*, *B* and *C* are the fitting parameters obtained by the regression of the experimental data. Tie-lines (TLs) for each phase diagram, i.e. the compositions of each phase for a common mixture composition, as well as the tie-line lengths (TLLs), were determined according to the method reported by Merchuk *et al.*⁴⁵

Application of ABS to purify violacein. After the determination of the binodal curves for the biphasic system of cholinium-based IL + Tween 20, a mixture point in the biphasic region common to all systems was selected. This was the system composed of 30 wt% Tween 20 + 40 wt% cholinium-based IL + 30 wt% violacein extract (obtained from solid–liquid extraction). The systems were gravimetrically prepared (within ±10^{−4} g) by adding the correct

amounts of surfactant, IL and water. The overall mixture was vigorously stirred and centrifuged (400g, 20min) to reach the equilibrium at 298 K. The two phases were separated and weighed (within $\pm 10^{-4}$ g) and the violacein content in each phase was evaluated through UV–visible spectrophotometry at 571 nm. The quantification of the contaminants, namely proteins, in each phase was assessed using the Pierce™ BCA Protein Assay Kit. The concentrations were calculated using the calibration curve previously determined in the UV–visible spectrophotometer. At least two independent ABS were prepared, both phases being quantified, as well as the respective blanks (systems in which no crude violacein extract was added) to guarantee the elimination of possible interferences of the phase-forming components on the quantification of violacein and contaminants.

The ABS performance in the purification of violacein was assessed by evaluation of the partition coefficients of violacein and the main contaminants, total proteins (TP), and the selectivity of each system. The extraction efficiency values of violacein and TP were also calculated.

The partition coefficients of violacein and TP, $K_{\text{violacein}}$ and K_{TP} respectively, were determined according to Eqns (3) and (4):

$$K_{\text{violacein}} = [\text{violacein}]_{\text{Tween 20-rich phase}} / [\text{violacein}]_{\text{IL-rich phase}} \quad (3)$$

$$K_{\text{TP}} = [\text{TP}]_{\text{Tween 20-rich phase}} / [\text{TP}]_{\text{IL-rich phase}} \quad (4)$$

where [violacein] and [TP] represent the concentrations of violacein and TP respectively in the Tween 20-rich and IL-rich phases. The selectivity was also calculated according to Eqn (5):

$$\text{selectivity} = K_{\text{violacein}} / K_{\text{TP}} \quad (5)$$

The extraction efficiencies of violacein and TP, $EE_{\text{violacein}}$ and EE_{TP} respectively, were calculated according to Eqns (6) and (7):

$$EE_{\text{violacein}} = ([\text{violacein}]_{\text{Tween 20}} \cdot V_{\text{Tween 20}}) / ([\text{violacein}]_{\text{Tween 20}} \cdot V_{\text{Tween 20}} + [\text{violacein}]_{[\text{Ch}]X} \cdot V_{[\text{Ch}]X}) \quad (6)$$

$$EE_{\text{TP}} = ([\text{TP}]_{\text{Tween 20}} \cdot V_{\text{Tween 20}}) / ([\text{TP}]_{\text{Tween 20}} \cdot V_{\text{Tween 20}} + [\text{TP}]_{[\text{Ch}]X} \cdot V_{[\text{Ch}]X}) \quad (7)$$

where [violacein] and [TP] represent the concentrations of violacein and TP respectively in the Tween 20-rich and [Ch]X-rich phases and V represents the volume of the phases.

Back-extraction. An array of different solvents, namely toluene, *n*-hexane, acetone, 1,2-pentanediol, 3-pentoxypentan-1,2-diol [5.0.0], 3-hexyloxypentan-1,2-diol [6.0.0], 1,3-dipropoxypropan-2-ol [3.0.3], 1:1 menthol/thymol, ethyl acetate and 2-methyl-TFH (supporting information Table S1), were tested on the back-extraction of violacein from the Tween 20-rich phase. After the ABS purification of violacein from the main contaminant proteins, the best system was tested for back-extraction. The violacein-rich phase was isolated and re-diluted to 1 mL using ultrapure water. The violacein-rich aqueous solution was mixed with the tested solvents at a solvent/sample phase ratio of 0.5. The solution was left to reach phase equilibrium for 5 min at room temperature and the violacein-rich phase was isolated. The success of violacein isolation was confirmed by UV–visible analysis. The organic solvent-rich phase was evaporated under reduced pressure and characterized by ^1H NMR.

RESULTS AND DISCUSSION

Cell disruption

The screening of various surface-active compounds (~ 0.025 wt% wet cells) was investigated. Figure 1 depicts the concentration of violacein released to the extracellular medium. The results of the surface-active disruption methodology were compared with two control solvents (water and ethanol) and other non-surfactant agents. A large array of solvents belonging to different classes (cationic, anionic and non-ionic), including ionic liquids (varying cations, anions and alkyl chain lengths for different cations), were evaluated.

The common surfactants tested in this work were pre-selected considering some works previously reported by us, where, from a large list, these were considered as the most efficient on cell disruption.^{42,46,47} The results presented in Fig. 1 show that the non-tensioactive ILs were unable to permeate the cell membrane, thus being ineffective in violacein release. This is probably due to the low ability of hydrophilic ILs to disrupt cell membranes, considering their low affinity to interact with the phospholipids composing the membranes, due to their hydrophobic nature.^{42,48} On the other hand, and as expected, most of the surfactant compounds, particularly the non-ionic surfactants, allowed the disruption of cell membranes, leading to violacein release to a larger extent than the control solvents. Several studies have suggested that the interactions between these amphiphilic compounds and cell membranes and their proteins promote cell changes, namely expansion and/or permeation, leading to cell disruption and the consequent release of intracellular material.^{42,49} As can be observed, the ability to induce these changes in cell permeability and/or to solubilize violacein is dependent on the surfactant type, which is demonstrated by the intensity in the lysate supernatant (supporting information Fig. S3). The cationic surfactants, and particularly the surfactant ILs, allowed the disruption of cell membranes.¹⁷ However, after an alkyl chain length of $n = 12$, there is effectively no change in the extraction performance for cationic surfactants. Overall, their ability to extract violacein was not much improved when compared with ethanol extraction. Nonetheless, $[\text{P}_{4,4,4,14}]\text{Cl}$ presented a much better performance than the other cationic surfactants, standing out as one of the best compounds to induce cell disruption. Among the studied compounds, the non-ionic surfactants were selected as the most efficient to permeate the cell membrane, leading to violacein release (purple bars in Fig. 1). The charge of the surfactant seems to have an important role in the definition of the best surfactant to promote extraction. Since the pH environment induced by the aqueous solutions of surfactant is close to neutral, violacein is mainly present in its non-ionic form.⁵⁰ This may lead to more stable interactions between the biomolecule and the micelle hydrophobic core, thus contributing to the increased solubility of violacein in the aqueous surfactant solutions. The results suggest that the best extractive solvents for violacein are the aqueous solutions of Tween 20 and Triton X-114, with the aqueous solution of Tween 20 representing the highest concentration of violacein extracted from the cells ($\sim 53\%$ more pigment extracted when compared with the extraction with ethanol).

After selecting the best compounds to promote the extraction of violacein, further studies were carried to understand the effect of consecutive extractions. The following extractions consisted in subjecting the biomass to a clean solvent solution repeatedly until no peak at 571 nm was observed. Considering Fig. 2, it is perceptible that the aqueous solution of Tween 20 displayed the best recovery. Indeed, most violacein was extracted on the first cycle, with only a small fraction of the pigment remaining in the

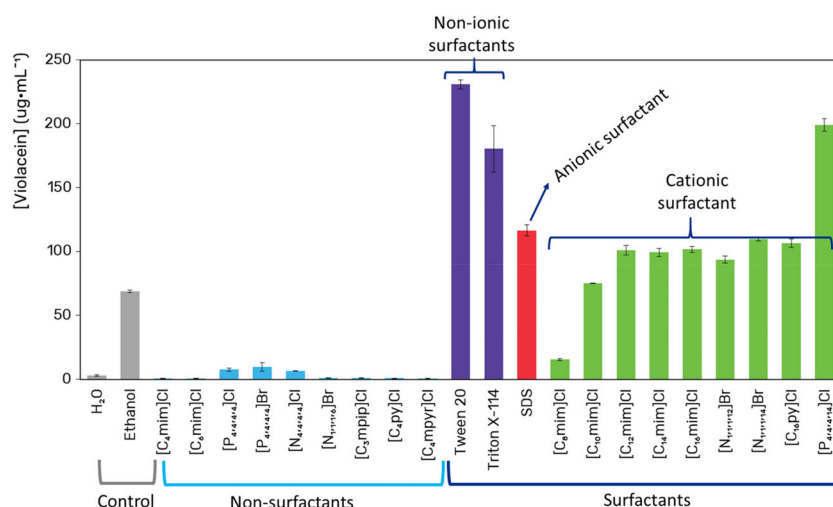


Figure 1. Concentration of violacein after single extraction using different solvents quantified at 571 nm: control solvents (grey) and aqueous solutions of non-surfactant compounds (light blue) and surfactant compounds as non-ionic surfactants (purple), anionic surfactants (red) and cationic surfactants (green). [Solvent] = 250 mmol L⁻¹, 30 min extraction, 50 rpm, 298 K.

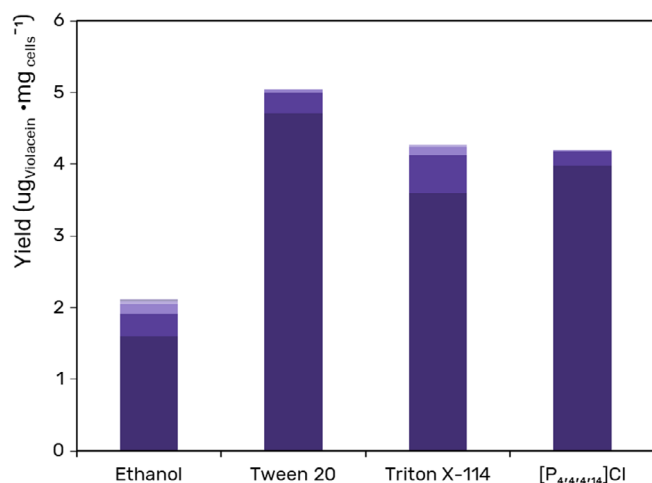


Figure 2. Concentration of violacein obtained from consecutive extractions using ethanol and aqueous solutions of Tween 20, Triton X-114 and [P_{4,4,4,14}]Cl as solvents. The extractions are displayed by order from first (bottom bar) to last (top bar), represented by darker to lighter purple respectively.

pellet (supporting information Fig. S4). Over 93% of violacein was recovered in a single-step extraction, and the remainder could be recovered during the consecutive extractions. None of the other solvents allowed for such a complete recovery of the pigment. Therefore the sequential extraction with these solvents did not extract all of the violacein content of these samples. Using this procedure, the quantities of violacein extracted with [P_{4,4,4,14}]Cl, Triton X-114 and ethanol were 85, 83 and 42% respectively. Ethanol showed the worst performance (Fig. 2 and supporting information Fig. S4). The extraction performance was best with the aqueous solution of Tween 20, so this was the solvent used in further studies.

Optimization of solid–liquid extraction

The effect of the process conditions, namely SLR, extraction time and solvent concentration, on the extraction yield of violacein was investigated using aqueous solutions of Tween 20, while ethanol

was used as control. Owing to the importance of the SLR in the extraction process,^{49,51,52} its effect was evaluated in the range 1:200–1:20 (mass of wet cells/volume of solvent) considering a 30 min extraction time. Figure 3A shows the yield of extraction with different SLRs. The yield of extraction decreased with increasing SLR until 0.025. Higher values of SLR did not significantly improve the extraction yield.

Lower SLRs present higher yields owing to the presence of more solvent per biomass, which, from the industrial point of view, is not always an advantage. Therefore, in this work, despite the higher values of violacein extraction obtained for lower SLRs, we selected 0.025 to proceed in the study, considering the balance between the yield of extraction of violacein and the amount of biomass possible to treat per mL of solvent.

A kinetic study was carried for 4 h considering an SLR of 0.025 (g wet cells mL⁻¹ solvent). The extraction results are depicted in Fig. 3B. The yield of extraction increased with the progression of time of extraction. The maximum of extraction was reached at 240 min for the aqueous solution of Tween 20, which makes it the time selected for further experiments. This happens considering the longer contact time between the solvent and the biomass, which promotes more interactions between the solvent and the cell membrane, which facilitates cell disruption. Hence more violacein is extracted and consequently better yields are attained.

The solvent concentration was also investigated considering an SLR of 0.025 (mass of wet cells/volume of solvent) and a 30 min extraction time. Four different concentrations of Tween 20 (50, 150, 250 and 325 mmol L⁻¹) were studied, the results being depicted in Fig. 4. The data obtained show the small impact of the surfactant concentration on the violacein extraction yield, since all concentrations of Tween 20 tested were well above its critical micelle concentration (CMC) of 0.06 mmol L⁻¹,⁵³ thus inhibiting micelle formation and decreasing the extraction yield of violacein.

Finally, at the end of the optimization of the solid–liquid extraction step, the viability of the cells exposed to Tween 20 aqueous solution and ethanol (control) was evaluated by their ability to re-grow in LB solid agar medium. As shown in supporting information Fig. S5, even after disruption of the cells with Tween 20 aqueous solution, they were able to re-grow, thus proving the

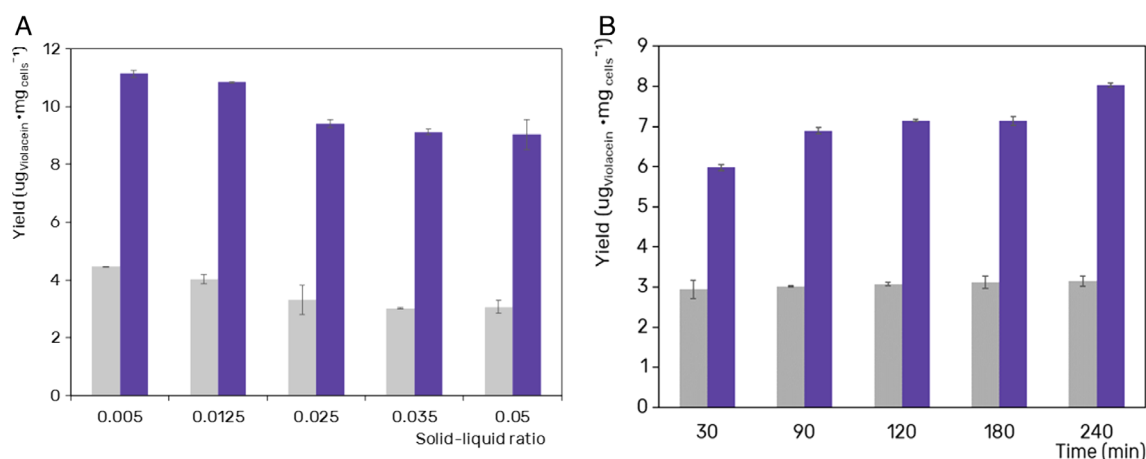


Figure 3. Yield of extraction of violacein for (A) different SLRs and (B) different times of extraction using ethanol (grey bars) and aqueous solution of Tween 20 (purple bars).

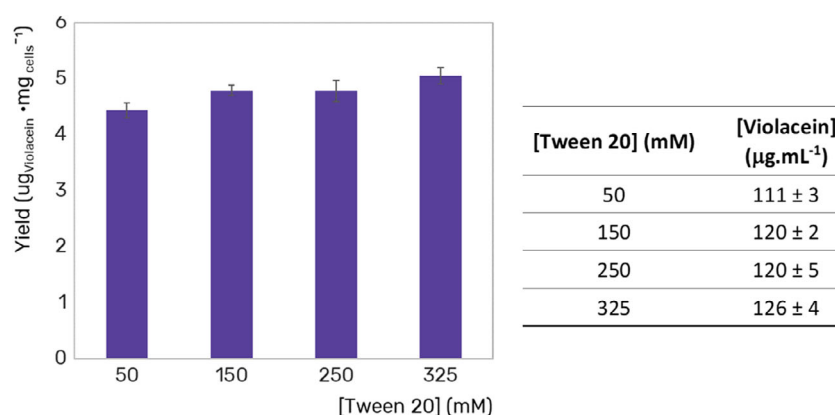


Figure 4. Variation of extraction yield of violacein (graph) and concentration of violacein (table) with different concentrations of Tween 20 in aqueous solution.

biocompatibility of Tween 20 towards the cells, contrary to what was observed for ethanol.

Violacein purification using ABS

In the previous section, the efficiency of Tween 20 aqueous solutions to extract violacein from *Y. lipolytica* cells was demonstrated. However, through an analysis of the UV-visible spectra, the presence of proteins was detected at 562 nm using the Pierce™ BCA Protein Assay Kit, suggesting contamination of the violacein-rich extract with proteins. A further step of purification using ABS is therefore proposed. ABS composed of Tween 20 + water + cholinium-based ILs were proposed in this work to promote the purification of violacein, separating it from the main contaminants.^{54,55} This IL family was selected owing to its lower cost and higher biocompatibility and sustainability.

Phase diagrams

Before the application of the selected ABS to the separation of violacein and proteins, five phase diagrams composed of Tween 20, cholinium-based ILs and water were determined at 298 K and atmospheric pressure, the respective binodal curves being illustrated in supporting information Fig. S6. The five ILs studied were [Ch][Ac], [Ch][DHP], [Ch][DHC], [Ch]Cl and [Ch][Bic]. The systems based on Tween 20 + [Ch][DHP], [Ch][Ac] and [Ch]Cl already reported in the literature^{54,55} were also considered for comparison.

All the detailed experimental data related (ternary phase diagrams weight fraction compositions, and equation 2 regression parameters) are reported in supporting information Tables S2–S8 and Fig. S7.

The binodal curves are plotted in supporting information Fig. S6 in molality units to eliminate effects coming from the differences in molecular weights of the cholinium-based compounds. In this figure, the effect of the IL anion on the ABS formation is evidenced. In all phase diagrams, the biphasic and monophasic regions are respectively placed above and below the solubility curve. Overall, it is possible to infer that the bigger the biphasic region, the better the capability of the cholinium-based compounds to induce liquid–liquid demixing. From the set of results, it is possible to conclude that [Ch][DHP] showed the highest ability to form ABS combined with Tween 20, whereas [Ch]Cl showed the weakest. The ability of the studied cholinium-based compounds to form ABS can be ranked as follows: [Ch][DHP] > [Ch][DHC] > [Ch][Ac] > [Ch][Bic] > [Ch]Cl. The tendency observed is in agreement with previous reports for ABS composed of [Ch]X and Pluronic L-35.⁵⁶

Purification of violacein

To evaluate the separation of violacein from contaminant proteins, a mixture point common to all tested systems, composed of 30 wt% Tween 20 + 40 wt% [Ch]Cl + 30 wt% aqueous solution of violacein raw extract, was applied. The previous results suggest

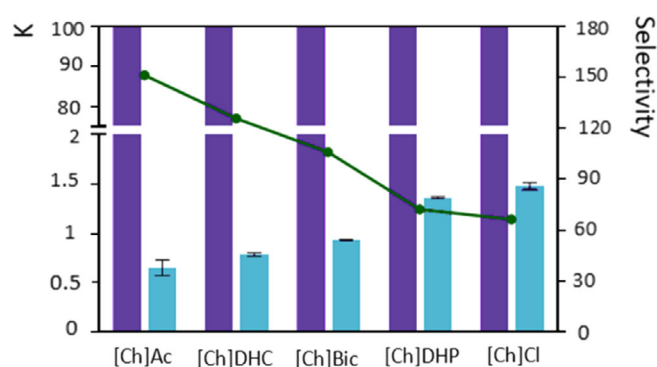


Figure 5. Influence of different [Ch]X on violacein partition coefficient (purple bars), total protein partition coefficient (blue bars) and selectivity of each system (green dots) in each ABS composed of Tween 20 + [Ch]X + water.

a high affinity of violacein for the most hydrophobic phase represented by the Tween 20 layer, which is also observed when ABS are applied ($K_{\text{violacein}} > 100$ and $EE_{\text{violacein}} \sim 100\%$, independently of the system used). Given the extensive partition of violacein towards the Tween 20-rich phase, the purification of violacein was evaluated by considering the maximization of the contaminants' presence in the opposite phase. In all ABS, the pigment stays concentrated in the most hydrophobic Tween 20-rich phase. Given the pH (7–10) of the systems studied, violacein is present in its non-ionic form. This lack of charge leads to favorable interactions between the biomolecule and the non-ionic surfactant.

By analyzing the results depicted in Fig. 5 and supporting information Fig. S8 (data of extraction efficiency represented), it is demonstrated that the contaminant protein partitioning depends on the nature of the cholinium anion and increases to the bottom phase according to the trend $[\text{Ch}]\text{Cl} < [\text{Ch}]\text{DHP} < [\text{Ch}]\text{Bic} < [\text{Ch}]\text{DHC} < [\text{Ch}]\text{Ac}$, which is also represented by the selectivity parameter. Proteins are composed of hydrophobic and hydrophilic amino acids, and this variety of polarities, along with a protein's net charge, controls its preferential partition to a given phase.

In this study, the systems showing the best purification capacity/selectivity are those with the more 'hydrophobic' anions, suggesting that the nature of the proteins allows an extensive separation of the contaminants from the violacein.

Back-extraction

In this study, a set of solvents was tested on the back-extraction of violacein from the Tween 20-rich phase. The major objective of this final step of back-extraction was, after removing the contaminant proteins, to separate the surfactant from the bioactive compounds extracted from the fresh biomass. The major outcomes are reported in supporting information Tables S2 – S8. Only ethyl acetate and 2-methyl-THF were successful in the back-extraction process. After back-extraction with these organic solvents, only traces of Tween 20 and [Ch][Ac] can be found in the recovered violacein, as depicted in supporting information Figs S9–S11. Despite the good results of both organic solvents, 2-methyl-THF is preferred, since it is industrially approved. Moreover, and since violacein is highly hydrophobic when compared with the remaining solvents involved in the whole process, at a larger scale, precipitation of violacein in cold water may be achieved. With this simple step, the purity of violacein can be greatly enhanced.

In the end, a final process was envisioned as depicted in Fig. 6. Here the violacein was efficiently extracted from the fresh cells by using Tween 20 aqueous solutions as the most efficient solvent, achieving a yield of extraction of $8 \mu\text{g}_{\text{violacein}} \text{mg}_{\text{cells}}^{-1}$. Despite the high yield, the selectivity was compromised by the presence of proteins, and thus a second step contemplating the separation of pigment from the contaminant proteins by applying ABS composed of Tween 20 + [Ch][Ac] + water was applied, making possible the recovery of violacein in a purer form, represented by the high selectivity (~ 155) obtained. As a last step, the polishing of both violacein from the Tween 20-rich phase and contaminant proteins from the [Ch][Ac]-rich phase was envisioned by applying two common strategies. On one hand, an ultrafiltration step may be applied to isolate the contaminant proteins from the [Ch][Ac]-rich phase, enabling its reintroduction in the purification

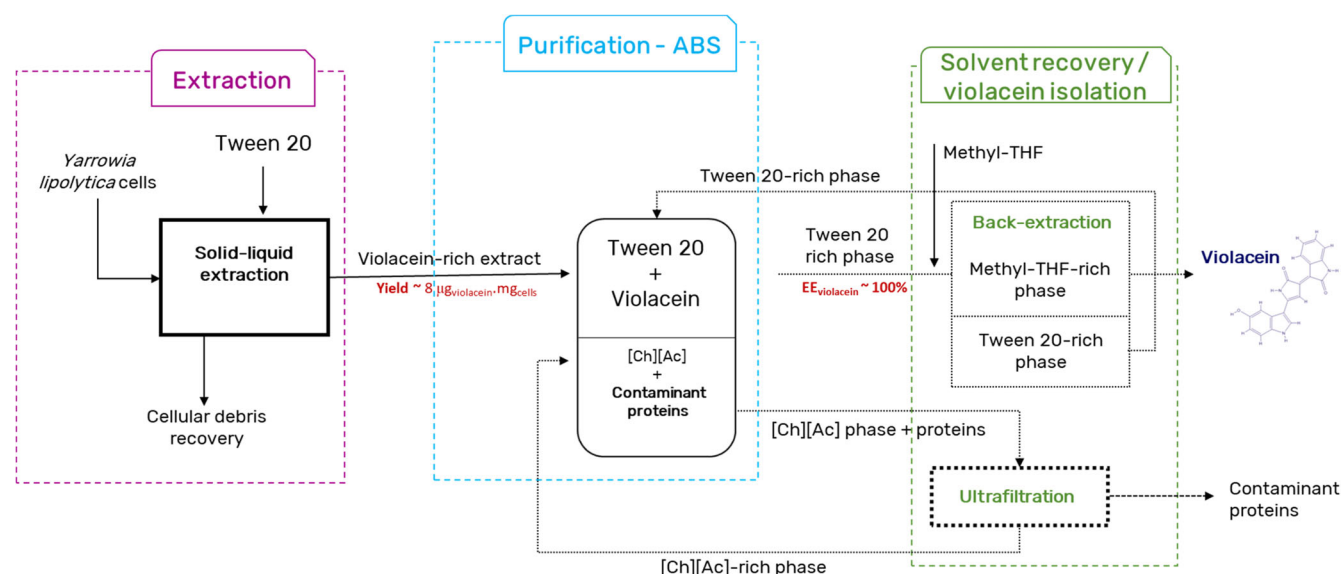


Figure 6. Schematic diagram of complete process envisioned in this work to extract and purify violacein from *Yarrowia lipolytica* cells. The recovery of the solvents is also contemplated. The dashed lines mean that the step was not performed experimentally but its application was previously reported as efficient in works with similar purposes.

unit. On the other hand, a back-extraction was performed to allow the separation of the pigment from the Tween-20-rich phase. In this case, considering that the violacein is here the target product, the back-extraction was experimentally performed by adding 2-methyl-THF and ethyl acetate, solvents with enough affinity for the pigment to concentrate and remove it from most contaminants. However, 2-methyl-THF was selected for this process, since its use is industrially approved. Again, the surfactants after polishing can be reintroduced in the first step of solid–liquid extraction.

CONCLUSION

The aim of this work was the optimization of the extraction of violacein from *Y. lipolytica* cells and later its fractionation from the proteins considered in this work as the main contaminants. It was possible to define an integrated process in which the violacein was efficiently extracted from the fresh cells by using Tween 20 aqueous solutions, followed by its separation from the main contaminants by applying an ABS composed of Tween 20 + [Ch][Ac] + water. In the end, the final downstream process was successfully envisioned, where the extraction of violacein was developed and this successfully separated from the contaminant proteins (maximum selectivity ~155) and further isolated from the main solvents (surfactant and [Ch][Ac]) through a back-extraction using 2-methyl-THF.

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Supporting Information

Supporting information may be found in the online version of this article.

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